REMARKS

Claims 1-29 were pending and were restricted as between the nucleic acid (claims 1 to 29) and protein (claims 1 to 29). Applicant elected the nucleic acid claims with traverse. Claims 5-7, 9, 12-15, 22, 25 and 26 have been withdrawn from consideration. Examined claims 1-4, 8, 10, 11, 16-21 and 27-29 stand rejected under 35 U.S.C. 112, first paragraph. Attached hereto is a copy of the currently pending claim set.

In view of the following remarks, Applicant respectfully requests reconsideration of the restriction requirement and of the application.

Restriction Requirement

The Examiner has reiterated the restriction requirement as between allegedly distinct two Groups. Both allegedly distinct Groups include all pending claims 1-29. Applicant again notes that the outstanding Restriction Requirement confuses restriction and election practice. How can claims be patentably distinct from themselves? At best, the claims are directed to patentably distinct species, namely, nucleic acids encoding chemokines and polypeptide chemokines. Thus, Applicant has elected claims directed to methods of enhancing an immune response by administering a nucleic acid encoding a chemokine. Upon allowance of a generic claim (*e.g.*, claim 1), Applicant will be entitled to consideration of claims to the additional species.

The Examiner has not indicated the restriction requirement is final and, for the reasons given above and those of record, Applicant submits that it should be withdrawn. Applicant again expressly reserves the right, pursuant to 35 USC §121, to file one or more divisional applications directed to the nonelected subject matter during the pendency of this application.

35 U.S.C. 112, First Paragraph, Enablement

Claims 1-4, 8, 10, 11, 16-21, 23-24 and 27-29 stand rejected under 35 U.S.C. 112, first paragraph as allegedly not enabled by the specification as filed. (Office Action, page 4). It is acknowledged that the specification teaches a route of delivery, dosage amount, frequency of administration, bleeding schedules with different plasmids

expressing non-structural HCV polypeptides and MIP-1α in baboons. Nonetheless, it is alleged that it would require undue experimentation to practice the claimed invention. In this regard, the Examiner cites several references (Mountain, Trepo, Howard and Lagging) in support of the allegation that gene therapy as a whole was unpredictable at the time of filing and that animal "models do not mimic relevant human conditions." (Office Action, page 7).

Applicant traverses each and every basis of this rejection and address them in turn.

The Specification Fully Enables the Claims Throughout their Scope

The test of enablement is whether one reasonably skilled in the art could make or use the invention from the disclosures in the patent coupled with information known in the art without undue experimentation. *Ex parte Forman*, 230 USPQ 546 (BPAI 1986). Whenever the PTO makes a rejection for failure to teach how to make and/or use the invention, the PTO must explain its reasons for the rejection and support the rejection with (i) acceptable evidence, or (ii) reasoning which contradicts the Applicant's claim: the reasoning must be supported by current literature as a whole and the PTO must prove the disclosure requires undue experimentation. *In re Marzocchi*, 439 F.2d 220, 223-24, 169 USPQ 367, 369-70 (CCPA 1971). It is well-settled that the enablement requirement is satisfied if the applicant's specification teaches one of skill in the art how to make and use the claimed invention without undue experimentation. *In re Wands,* 8 USPQ2d 1400 (Fed. Cir. 1988). When determining whether the amount of testing required is "undue," the courts have determined that "time and difficulty of experiments are not determinative if they are merely routine." (see, *e.g., In re Wands,* 8 USPQ2d at 1404, citing *In re Angstadt*, 190 USPQ 214 (CCPA 1976).

Applicant also notes the "invention" referred to in the enablement requirement of section 112 is the claimed invention." *See, Christianson v. Colt Industries Operating Corp.* 3 USPQ2d 1241 (Fed. Cir. 1987), emphasis added. Thus, the Office must first determine what each claim recites when the claim is considered as a whole, not when its parts are analyzed individually. See, Training Manual on Enablement, page 9. Moreover,

the existence of inoperative or ineffective embodiments does not mean that the enablement requirement is not satisfied. Indeed, if any use of multiple uses disclosed in the specification are enabled, the application is enabling. See, Training Manual, page 21.

As a threshold matter, Applicant notes that only examined claims 11, 16-21, 23, 24 and 27-29 are directed to methods of generating an immune response and none of these claims recite vaccine compositions or methods of treating (or vaccinating against) a DNA immunogen. Rather, they are drawn to compositions comprising particular sequences or to methods of enhancing an immunological response in a mammal using these compositions. As is well-known and described, for example, on page 4, lines 13-19 of the specification, an immunological response can be either a humoral immune response (*e.g.,* mediated by antibodies) or a cellular immune response (*e.g.,* mediated by T-lymphocytes and/or other white blood cells). Therefore, when properly interpreted in light of the specification, the pending claims are directed to methods of eliciting cellular and/or humoral immune responses and the enablement requirement is satisfied by Applicant's showing that these methods elicit such immune responses. (See, Examples).

Turning to the rejection itself, the Examiner has acknowledged that the specification enables a skilled artisan to practice the claimed invention using HCV immunogens and a polynucleotide encoding MIP-1α in baboons. (Office Action, page 4). However, it is still asserted that the specification lacks sufficient guidance as to DNA immunogens other than HCV non-structural polypeptides; chemokines other than MIP-1α and subjects other than baboons. This is not a correct application of the law and, moreover, completely refuted by the evidence of record. Applicant is under no legal obligation to specifically recite (or exemplify) each and every DNA immunogen, each and every chemokine or each and every potential subject. Rather, the application need only disclose to one of skill in the art how to identify and use such immunogens, chemokine-encoding polynucleotides and subjects without undue experimentation. The specification clearly satisfies this requirement.

The specification clearly teaches how to identify and use other DNA immunogens. For instance, useful DNA immunogens disclosed by Applicant include viral polypeptides (HIV gag, pol, env; herpes viruses, EBV, HBV, HCV, HPV), as well as

immunogens from bacteria, fungi, yeast and tumor polypeptides. (see, page 5, lines 14-26 of the application). The specification also teaches that more than one immunogenic polypeptide can be used. (See, page 5, lines 25-26). The specification actually exemplifies both HIV and HCV DNA immunogens. (See, *e.g.*, Example 2 disclosing that co-administration of HIV/BLC increases titers of the HIV antibodies). Following the teachings of the specification, one of skill in the art could readily identify and use any DNA immunogen.

Similarly, suitable chemokines are discussed, for example, on page 4, lines 3-12 and MIP-1α and BLC are actually exemplified. The specification also teaches how to test such immunogens/chemokine compositions for immunogenicity and how to use these compositions in any subject. In other words, it would require only routine experimentation for a skilled artisan to follow the teachings of the specification to select any DNA immunogen, any chemokine-encoding polynucleotide and use these in any subject to enhance the immune response to the immunogen.

In sum, despite the Office's failure to establish a *prima facie* case of non-enablement, Applicant has established that the specification fully enables the pending claims by enabling not only a single use, but by enabling the claims throughout their scope.

In vivo Mouse and Baboon Models

The Examiner alleges that there is no established correlation between the animal models used in the Examples and the subject matter of the examined claims, directed to immunogenic compositions and methods of nucleic acid immunization which result in an enhanced immune response to a DNA immunogen.

Applicant strongly disagrees with the Examiner's position. It is well-settled that the existence of a reasonable "correlation" is dependent on the state of the art and that the burden is on the Examiner to determine whether a skilled artisan would accept the model used as reasonably correlating to the claims. *In re Brana*, 34 USPQ2d 1436, 1441 (Fed. Cir. 1995). Moreover, a rigorous or an invariable exact correlation is not required. *Cross v. Iizuka*, 224 USPQ 739, 747 (Fed. Cir. 1985). Thus, the specification need only

provide evidence which reasonably establishes that the claimed compositions and methods are enabled.

In the pending application, Applicant's *in vivo* data establish enablement of the claimed compositions and methods. With regard to the mouse data, Applicant notes that the mouse model described in the Examples is well-accepted by those skilled in the art as reasonably correlating to human responses. Indeed, it has long been established in the field of immunology that the mouse model is one of the most appropriate animal models for evaluating immune responses. In 1996, when the Nobel Prize was awarded to mouse immunologists Peter Doherty and Rolf Zinkernagel for their work in this area, the Nobel Committee specifically recognized the importance of the mouse model in laying "a foundation for an understanding of general mechanisms used by the cellular immune system." (See, Exhibit A). The Nobel Committee noted that the mouse model is "highly relevant to clinical medicine." (see, Exhibit A, Summary, attached hereto). The Nobel Committee went on to state that:

> "Many common and severe diseases depend on the function of the cellular immune system and consequently on its mechanism for specific recognition. Although this naturally applies to infectious diseases, this is also true of a number of chronic inflammatory conditions such as rheumatic diseases, diabetes and multiple sclerosis. Where infectious diseases are concerned, the new knowledge provides a better platform for the construction of new vaccines; one can ascertain exactly what parts of a microorganism are recognized by the cellular immune system, and can specifically focus the production of the vaccine on those parts. Furthermore, regard is paid to the fundamental principles formulated by Doherty and Zinkernagel in trials with vaccinations against the emergence of metastases in certain forms of cancer...." (Exhibit A, page 3).

Similarly, the baboon is a widely acceptable model for studying immune responses to immunogens, such as viral antigens. (See, Exhibit B, Locher et al. (1998); Klinger et al. (1998); Jenson et al. (2000); Lalain et al. (2001); Kalter et al. (1983); and Locher et al. (2001)).

Thus, the state of the art at the time of filing plainly evidences that experiments performed in mice and baboons to evaluate immune responses in response to nucleic acid immunization would reasonably correlate and reasonably predict human

immunological responses to DNA immunogens (such as those derived from infectious virally-transmitted diseases such as hepatitis, HIV and the like). In addition, the evidence establishes that the mouse and primate models are also useful in the development of new vaccines for disease which involve an immune response.

In view of this evidence, it is clear that the data presented by Applicant reasonably correlates to the full scope of the claims. Accordingly, the specification as filed fully enables the pending claims and withdrawal of this rejection is requested.

The Cited References Do Not Establish Unpredictability

Applicant also traverses the Examiner's assertion that the references establish that the claimed invention is unpredictable. (Mountain, Trepo, Howard and Lagging, cited on pages 5-6 of the Office Action).

Applicant requests clarification as to what the Examiner regard as the "Mountain" and "Trepo", as these two references were not included in the 1449 form attached to the Office Action. Even assuming that the Examiner meant to reference the citations attached to the Office Action, none establish unpredictability of the claimed invention. Indeed, Inchauspé et al. (1998) report "successful induction of both [humoral and cellular] types of responses in a murine model following immunisation with a panel of C and E2 encoding plasmids." (See, Introduction). Howard et al report that HCV antigens is presented with high efficiency following DNA injection and offers the potential of high rates of seroconversion and virus clearance..." (See, Abstract). Nakano et al. (1997) is directed to the identification of immunogenic domains with E2 and indicates that different routes of administration can give different humoral immune responses. (See, Abstract). Notably, cellular immune responses were not tested and, moreover, all routes of injection resulted in generation of antibodies. (See, Figures). Similarly, Lagging reports that administration of plasmid DNA encoding HCV core proteins generated "antibody responses, lymphoproliferative responses and cytotoxic T-lymphocyte activity." (See, Abstract). Breitburd et al. (1999) is directed to generation of an HPV vaccine. Walther et al. (2000) is a review article directed solely to viral vectors. Thus, these references are a far cry away from establishing that methods of eliciting

immune responses are not enabled by Applicant's specification. In fact, Applicant's specification describes and demonstrates the generation of an immune response and, accordingly, the various references cited by the Office are not relevant to the claimed invention and certainly do not establish unpredictability of the claimed invention.

For the all the foregoing reasons, Applicant submits that the specification fully enables the claims and respectfully requests withdrawal of this rejection.

CONCLUSION

For the reasons state above, Applicant respectfully submits that the pending claims define an invention which is novel and fully enabled by the specification. Accordingly, Applicant requests that the rejection of the claims be withdrawn, and that the application proceed to allowance.

The Commissioner is hereby authorized to charge any fees under 37 C.F.R. §§ 1.16 and 1.17 which may be required by this paper, or to credit any overpayment, to Deposit Account No. 18-1648.

Please direct all further communications regarding this application to:

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Currently Pending Claims

1. An immunogenic composition comprising:
a DNA immunogen; and
a chemokine or a polynucleotide encoding a chemokine.

2. The immunogenic composition of claim 1 wherein the DNA immunogen comprises a polynucleotide encoding a viral immunogen.

3. The immunogenic composition of claim 2 wherein the polynucleotide encodes a hepatitis C virus non-structural polypeptide.

4. The immunogenic composition of claim 3 wherein the hepatitis C virus non-structural polypeptide is selected from the group consisting of NS3, NS4, NS5a, and NS5b.

5. (Withdrawn) The immunogenic composition of claim 2 wherein the polynucleotide encodes an HIV polypeptide.

6. (Withdrawn) The immunogenic composition of claim 5 wherein the HIV polypeptide is a gag polypeptide.

7. (Withdrawn) The immunogenic composition of claim 1 wherein the DNA immunogen comprises a polynucleotide encoding an immunogen expressed by a tumor.

8. The immunogenic composition of claim 1 wherein the chemokine is macrophage inflammatory protein 1α (MIP-1α).

9. (Withdrawn) The immunogenic composition of claim 1 wherein the chemokine is B lymphocyte chemokine (BLC).

10. The immunogenic composition of claim 1 further comprising a pharmaceutically acceptable carrier.

11. A method of enhancing an immune response to a DNA immunogen in a mammal comprising the step of:
administering to the mammal (i) a chemokine or a first polynucleotide encoding a chemokine and (ii) a DNA immunogen, whereby an immune response to the DNA immunogen is enhanced.

12. (Withdrawn) The method of claim 11 wherein a chemokine is administered.

13. (Withdrawn) The method of claim 12 wherein the chemokine and the DNA immunogen are co-administered.

14. (Withdrawn) The method of claim 12 wherein the chemokine is administered prior to the administration of the DNA immunogen.

15. (Withdrawn) The method of claim 12 wherein the DNA immunogen is administered prior to administration of the chemokine.

16. The method of claim 11 wherein the first polynucleotide encoding the chemokine is administered.

17. The method of claim 16 wherein the first polynucleotide and the DNA immunogen are co-administered.

18. The method of claim 16 wherein the polynucleotide is administered prior to the administration of the DNA immunogen.

19. The method of claim 16 wherein the DNA immunogen is administered prior tot he administration of the first polynucleotide.

20. The method of claim 16 wherein a second polynucleotide which comprises (a) the first polynucleotide and (b) the DNA immunogen is administered.

21. The method of claim 11 wherein the chemokine is macrophage inflammatory protein 1α (MIP-1α).

22. (Withdrawn) The method of claim 11 wherein a chemokine is B lymphocyte chemokine (BLC).

23. The method of claim 11 wherein the DNA immunogen comprises a polynucleotide encodes a hepatitis C virus non-structural polypeptide.

24. The method of claim 23 wherein the hepatitis C virus non-structural polypeptide is selected from the group consisting of NS3, NS4, NS5a, and NS5b.

25. (Withdrawn) The method of claim 23 wherein the polynucleotide encodes an HIV polypeptide.

26. (Withdrawn) The method of claim 25 wherein the HIV polypeptide is a gag polypeptide.

27. The method of claim 11 wherein the mammal is human.

28. The method of claim 11 wherein the immune response is an antibody response.

29. The method of claim 11 wherein the immune response is a cytotoxic T lymphocyte response.

EXHIBIT A

Press Release: The 1996 Nobel Prize in Physiology or Medicine

NOBELFÖRSAMLINGEN KAROLINSKA INSTITUTET
THE NOBEL ASSEMBLY AT THE KAROLINSKA INSTITUTE

7 October 1996

The Nobel Assembly at the Karolinska Institute has today decided to award the Nobel Prize in Physiology or Medicine for 1996 jointly to

Peter C Doherty and Rolf M Zinkernagel

for their discoveries concerning "the specificity of the cell mediated immune defence".

Summary

Peter Doherty and Rolf Zinkernagel have been awarded this year's Nobel Prize in Physiology or Medicine for the discovery of how the immune system recognizes virus-infected cells. Their discovery has, in its turn, laid a foundation for an understanding of general mechanisms used by the cellular immune system to recognize both foreign microorganisms and self molecules. This discovery is therefore highly relevant to clinical medicine. It relates both to efforts to strengthen the immune response against invading microorganisms and certain forms of cancer, and to efforts to diminish the effects of autoimmune reactions in inflammatory diseases, such as rheumatic conditions, multiple sclerosis and diabetes.

The two Nobel Laureates carried out the research for which they have now been awarded the Prize in 1973-75 at the John Curtin School of Medical Research in Canberra, Australia, where Peter Doherty already held his position and to which Rolf Zinkernagel came from Switzerland as a research fellow. During their studies of the response of mice to viruses, they found that white blood cells (lymphocytes) must recognize both the virus and certain self molecules - the so-called major histocompatibility antigens - in order to kill the virus-infected cells. This principle of simultaneous recognition of both self and foreign molecules has since then constituted a foundation for the further understanding of the specificity of the cellular immune system.

The background to the Laureates' research

The immune system consists of different kinds of white blood cells, including T- and B-

lymphocytes whose common function is to protect the individual against infections by means of eliminating invading microorganisms and infected cells. At the same time they must avoid damaging the own organism. What is required is a well developed recognition system that enables lymphocytes to distinguish between on the one hand microorganisms and infected cells, and on the other, the individual´s normal cells. In addition, the recognition system must be able to determine when white blood cells with a capacity to kill should be activated.

In the early 1970s when Peter Doherty and Rolf Zinkernagel had begun their scientific work within immunology, it was possible to distinguish between antibody-mediated and cell- mediated immunity. It was known that antibodies that are produced by B-lymphocytes are able to recognize and eliminate certain microorganisms, particularly bacteria. Far less was known about recognition mechanisms in the cellular immune system, for instance in conjunction with the killing of virus-infected cells by T-lymphocytes. One area where cellular immunity had previously been studied in some detail was, however, transplantation biology. It was known that T-lymphocytes could kill cells from a foreign individual after recognition of certain molecules - the major histocompatibility antigens - in the transplant.

The discovery

Rolf Zinkernagel and Peter Doherty used mice to study how the immune system, and particularly T -lymphocytes, could protect animals against infection from a virus able to cause meningitis. Infected mice developed killer T-lymphocytes, which in a test-tube could kill virus- infected cells. But there was an unexpected discovery: the T-lymphocytes, even though they were reactive against that very virus, were not able to kill virus-infected cells from another strain of mice. What decided whether or not a cell was eliminated by these killer lymphocytes was not only if they were infected with the virus, but also if they carried the "correct" variant of histocompatibility antigens, those of the infected mouse itself. Zinkernagel's and Doherty's findings, which were published in Nature in 1974 (1,2), demonstrated conclusively the requirement for the cellular immune system to recognize simultaneously both 'foreign' molecules (in the present case from a virus) and self molecules (major histocompatibility antigens). What also became obvious was the important function of the major histocompatibility antigens (in man called HLA-antigens) in the individual´s normal immune response and not only in conjunction with transplantation.

The discovery has given an impetus to later research

Zinkernagel's and Doherty´s findings had an immediate impact on immunological research. The wide relevance of their observations concerning the specificity of the T-lymphocytes became apparent in many contexts, both in regard to the ability of the immune system to recognize microorganisms other than viruses, and in regard to the ability of the immune system to react against certain kinds of self tissue. To explain their findings, the two scientists subsequently devised two models; one model was based on a single recognition of 'altered self''(when the histocompatibility antigen has been modified through association with a virus), the other on a 'dual recognition' of both foreign and self. (Fig.) Both the experimental findings and the theoretical models became immensely important in later research. Within a few years, it had been demonstrated that the set of the T- lymphocytes that are allowed to mature and survive in an individual is determined by the ability of the cell to recognize the transplantation antigens of the individual. Therefore, the principle of simultaneous recognition is essential for the ability of the immune system to distinguish between 'self' and 'non-self'.

Further molecular research has both confirmed Zinkernagel's and Doherty's models and

clarified the structural basis of their discovery - that a small part (a peptide), for example from a virus, is directly bound to a defined variable part of the body's own histocompatibility antigens, and that this complex is what is recognized by the specific recognition molecules of T- lymphocytes (T-cell receptors). Taken in all, the clarification of the recognition mechanisms of the T-cells within the cellular immune system has fundamentally changed our understanding of the development and normal function of the immune system and, in addition, has also provided new possibilities for the selective modification of immune reactions both to microorganisms, and to self tissues.

Relevance for clinical medicine

Many common and severe diseases depend on the function of the cellular immune system and consequently on its mechanisms for specific recognition. Although this naturally applies to infectious diseases, this is also true of a number of chronic inflammatory conditions such as rheumatic diseases, diabetes and multiple sclerosis. Where infectious diseases are concerned, the new knowledge provides a better platform for the construction of new vaccines; one can ascertain exactly what parts of a microorganism are recognized by the cellular immune system, and can specifically focus the production of the vaccine on those parts. Furthermore, regard is paid to the fundamental principles formulated by Doherty and Zinkernagel in trials with vaccination against the emergence of metastases in certain forms of cancer. In many chronic inflammatory diseases, better explanations have been provided for the associations between disease susceptibility and the histocompatibility antigen type carried by an individual. The research that followed from the now awarded discovery has also provided openings for selectively diminishing or altering immune reactions that play a central role in inflammatory diseases.

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Exhibit A

CD8 Positive T Cells Influence Antigen-Specific Immune Responses through the Expression of Chemokines

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Abstract

The potential roles of $CD8^+$ T-cell–induced chemokines in the expansion of immune responses were examined using DNA immunogen constructs as model antigens. We coimmunized cDNA expression cassettes encoding the α-chemokines IL-8 and SDF-1α and the β-chemokines MIP-1α, RANTES, and MCP-1 along with DNA immunogens and analyzed the resulting antigen-specific immune responses. In a manner more similar to the traditional immune modulatory role of $CD4^+$ T cells via the expression of Th1 or Th2 cytokines, $CD8^+$ T cells appeared to play an important role in immune expansion and effector function by producing chemokines. For instance, IL-8 was a strong inducer of $CD4^+$ T cells, indicated by strong T helper proliferative responses as well as an enhancement of antibody responses. MIP-1α had a dramatic effect on antibody responses and modulated the shift of immune responses to a Th2-type response. RANTES coimmunization enhanced the levels of antigen-specific Th1 and cytotoxic T lymphocyte (CTL) responses. Among the chemokines examined, MCP-1 was the most potent activator of $CD8^+$ CTL activity. The enhanced CTL results are supported by the increased expression of Th1 cytokines IFN-γ and TNF-α and the reduction of IgG1/IgG2a ratio. Our results support that $CD8^+$ T cells may expand both humoral and cellular responses in vivo through the elaboration of specific chemokines at the peripheral site of infection during the effector stage of the immune response. (*J. Clin. Invest.* 1998. 102:1112–1124.) Key words: chemokines • DNA immunization • $CD8^+$ T effector cells • humoral and cellular immune response

Introduction

The adaptive immune response is a critical part of host defense against pathogens. The immune response is initiated when local inflammation induces tissue macrophages to produce proinflammatory cytokines and chemokines. Collectively, these molecules recruit more phagocytic cells and professional antigen-presenting cells (APCs)[1] to the site of infection. Once attracted to the infection site, APCs ingest pathogenic antigens and transport them to local lymphoid organs. In the lymphoid organs, APCs process and present these antigens to naive T cells. When activated, $CD4^+$ T helper cells modulate the level and the direction of immune response through the release of Th1- or Th2-type cytokines. T helper cells activate B lymphocytes to produce antigen-specific antibodies, which engages the humoral response. T helper cells also help cytotoxic T lymphocytes to search out and destroy infected cells in the periphery. Once engaged, humoral or cellular immunity can act independently or in concert to eradicate the pathogenic organism from the host.

Although the importance of modulatory signals from T helper cells in directing the immune response is well appreciated, the role of signaling from $CD8^+$ T cells is not known. Cytotoxic T cells bind and destroy allogeneic and virally infected cells that display recognizable antigen-MHC class I molecules. Killer T cells induce these pathogen-infected cells to die through either the release of toxic proteins such as granzyme B or through initiating apoptosis or programmed cell death in the target cells. In addition to the direct killing of the infected cells, $CD8^+$ T cells may provide additional signals to other lymphocyte subsets to help amplify and direct the immune response in the periphery. These signals importantly might include the highly bioactive inflammatory molecules termed chemokines. Chemokines play a major role in a variety of immune and inflammatory responses, acting primarily as chemoattractants and activators of various leukocytes (1, 2). For example, in response to pathogens, tissue macrophages initiate host immune responses by producing chemokines. These chemokines in turn attract more leukocytes to the site of infection and tissue damage.

Chemokines are broadly divided into three families, C-X-C (α), C-C (β), and C (γ), based on the presence and position of the conserved cysteine residues (3). In the members of the α family, the first two cysteines are separated by another amino acid, while those of the β family are placed next to each other (3). Only two members of the γ family have been identified so far, and both of them contain one instead of two cysteines in their N terminus (3). Overall, there are many chemokines with seemingly overlapping functions, and the exact role of each one in host defense and in pathological responses is not well known. Recently the role of $CD8^+$ T cells in the production of chemokines has been appreciated (4–8). Their additional function in such immune expansion is unclear.

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1. *Abbreviations used in this paper:* APC, antigen-presenting cells; CTL, cytotoxic T lymphocyte; NK, natural killer; RD, rhabdomyosarcoma.

During evaluation of host immune responses induced by DNA vaccines for HIV-1 antigens, we observed that in addition to eliciting both humoral and cell-mediated immune responses, DNA vaccination induced β-chemokine expression in $CD8^+$ T lymphocytes. The temporal nature of these results implied that chemokines may be important activators of immune responses. To molecularly dissect the specific roles of chemokines in immune response, we cloned representative cDNAs encoding the α-chemokines IL-8 and SDF-1α as well as cDNAs encoding the β-chemokines MIP-1α, RANTES, and MCP-1. These inserts were cloned individually into expression vectors and coimmunized along with DNA immunogens encoding for HIV-1 envelope or gag/pol antigens. These chemokines are especially relevant since they are produced by activated T lymphocytes. Using these vaccine constructs as model antigens, we observed that individual chemokines had specific, identifiable roles in the activation and modulation of antigen-specific immune responses. The observation that $CD8^+$ effector cells elevated chemokine expression levels while they primed immune responses suggests a regulatory role for these end-stage effector cells in the expansion phase of an antigen-specific immune response. These results conceptually link lymphocyte activation and expansion driven by $CD4^+$ T cells within the lymphoid compartments such as the lymph nodes and spleen with lymphocyte expansion, tissue invasion, and effector function modulated at least in part by $CD8^+$ effector T cells in the periphery.

Methods

DNA plasmids. DNA vaccine constructs expressing HIV-1 envelope protein (pCEnv) and gag/pol protein (pCGag/Pol) were prepared as previously described. (9). The genes for human chemokines IL-8, SDF-1α, MIP-1α, MCP-1, and RANTES were cloned into the pCDNA3 expression vector (Invitrogen, Inc., San Diego, CA) as previously described (10, 11). Clean plasmid DNA was produced in bacteria and purified using Qiagen Maxi Prep kits (Qiagen, Santa Clara, CA).

Reagents and cell lines. Human rhabdomyosarcoma (RD) and mouse mastocytoma P815 cell lines were obtained from ATCC (Rockville, MD). Recombinant vaccinia expressing HIV-1 envelope (vMN462), gag/pol (vVK1), and β-galactosidase (vSC8) were obtained from the NIH AIDS Research and Reference Reagent Program. HIV-1 envelope peptide (RIHIGPGRAFYTTKN) was synthesized according to the previously published protocol (12). Recombinant gp120 or p24 protein were obtained from ImmunoDiagnostics, Inc. (Bedford, MA).

DNA inoculation of mice. The quadriceps muscles of 6–8-wk-old female BALB/c mice (Harlan Sprague Dawley, Inc., Indianapolis, IN) were injected with 50 μg of each DNA construct of interest formulated in PBS and 0.25% bupivacaine-HCl (Sigma Chemical Co., St. Louis, MO). Coadministration of various gene expression cassettes involved mixing the chosen plasmids before injection. The control mice were immunized with 50 μg of pCDNA3 vector. Each set of studies was performed three times, and a representative set of results is presented. Mice received two DNA immunizations (50 μg each) separated by 2 wk. At 1 wk after the boost injection, the mice were killed, the spleens were harvested, and the lymphocytes were isolated and tested for cellular (Th or cytotoxic T lymphocyte [CTL]) responses. All animals were housed in a temperature-controlled, light-cycled facility at the University of Pennsylvania, and their care was under the guidelines of the National Institutes of Health and the University of Pennsylvania.

In vivo expression of chemokine gene constructs. The quadriceps muscles of mice were injected with 50 μg of MIP-1α, RANTES, MCP-1, SDF-1α, and irrelevant control constructs formulated in PBS and 0.25% bupivacaine-HCl. The mice were killed and their leg muscle was removed 3 d after injection. The muscle was dissected, minced, and placed in tissue culture for 3 additional days. The supernatant was collected and tested for chemokine expression using the ELISA kit for MIP-1α, RANTES, and MCP-1 (Intergen, Purchase, NY).

ELISA. ELISA for mouse antisera was performed as previously described (10). For the determination of relative levels of gp120-specific IgG subclasses, antimurine IgG1 and IgG2a conjugated with HRP (Zymed, San Francisco, CA) were substituted for antimurine IgG-HRP. This was followed by addition of the ABTS substrate solution (Chemicon, Temecula, CA).

T helper cell proliferation assay. T helper cell proliferation assay was performed as previously described (10).

Cytotoxic T lymphocyte assay. A 5-h ^{51}Cr release CTL assay was performed using vaccinia-infected targets or peptide-treated targets as previously described (10).

Complement lysis of $CD8^+$ T cells. Complement lysis of $CD8^+$ T cells was performed as previously described (10).

Cytokine and chemokine expression analysis. Supernatants from effectors stimulated for CTL assay were collected at day 6 and tested for cytokine profile using ELISA kits for IFN-γ, IL-4, and TNF-α (Biosource International, Inc., Camarillo, CA). Supernatants from stimulated effector cells were also tested for chemokine profile using chemokine ELISA kits for MIP-1α (R&D Systems, Minneapolis, MD), RANTES, and MCP-1 (Intergen).

Results

Induction of chemokines by DNA vaccination. Mice were immunized with 50 μg of pCDNA3 (control), pCEnv, or pCGag/pol. After 2 wk, animals were killed, their spleens were harvested, and their lymphocytes were isolated and stimulated in a standard CD8 effector CTL assay (10, 11, 13). We collected the culture supernatant from the cultures and tested them for the release of chemokines MIP-1α, MIP-1β, and RANTES. We observed that DNA immunization with pCEnv or pCGag/pol induced significantly greater levels of expression of β-chemokines MIP-1α, MIP-1β, and RANTES over those of control vector as shown in Fig. 1 *A–C*, respectively. MIP-1α, MIP-1β, and RANTES were increased three- to fivefold. The increase was present as early as 2 wk after the first immunization and coordinated with the observed T cell and humoral responses, suggesting that these chemokines could be modulating immune responses in vivo. To determine the effects of the chemokines on antigen-specific responses, we next investigated their effects on immune responses induced by the model DNA vaccine.

Construction of chemokine expression cassettes. The cDNAs for human chemokines IL-8, SDF-1α, MIP-1α, MCP-1, and RANTES were individually cloned into pCDNA3 plasmid expression vectors by using methods previously described (10, 11, 13). Human SDF-1α, MIP-1α, MCP-1, and RANTES have been reported to be active in mouse cells (14). The mouse homologue of human IL-8 has not been found; however, human IL-8 has been reported to have activity on mouse cells as a subset of the receptors for IL-8 is expressed on mouse cells (14). These chemokine expression cassettes were verified by sequencing analysis of the entire insert (including both 5′ and 3′ flanking sequences). In addition, chemokine constructs were transfected in vitro into RD cells, and the expression of these constructs was verified by immunoprecipitation using relevant antibodies or by specific chemokine ELISA (data not shown). The expression constructs for IL-8, SDF-1α, MIP-1α, MCP-1, and RANTES were also tested for in vivo expression in mouse muscle. As presented in Fig. 2, the constructs expressed their

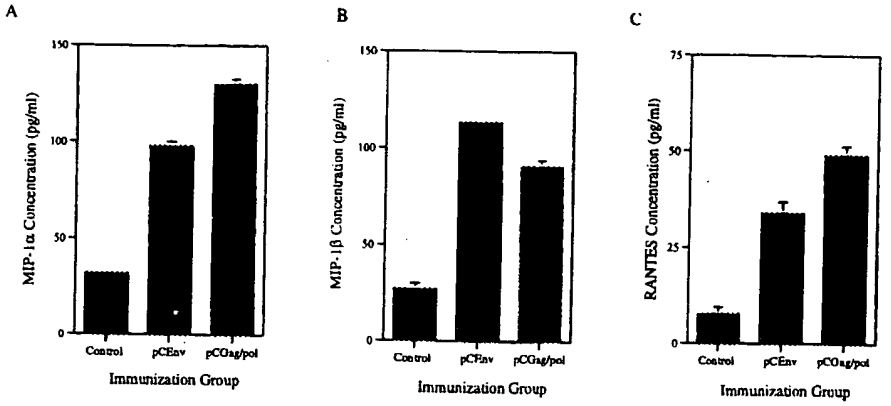

Figure 1. Induction of chemokines by DNA vaccination. Mice were immunized with 50 μg of pCDNA3 (*Control*), pCEnv, or pCGag/pol. After 2 wk, the mice were killed, the spleens were harvested, and the lymphocytes were isolated. These cells were stimulated in vitro with specific stimulation (using vaccinia infected stimulators) for 5 d. The culture supernatants from the effector cells were collected and were tested for the release of MIP-1α (*A*), MIP-1β (*B*), and RANTES (*C*).

respectively encoded chemokines in mouse muscle tissue in vivo at 3 d after transfection. Mice were immunized intramuscularly with 50 μg of pCMIP-1α or pCRANTES or pCDNA3 (control). The mice were killed, and their leg muscle was removed 3 after injection. The muscle tissues were mechanically pulverized and were cultured in vitro for 72 h and the supernatants were tested for the expression using the ELISA kits for MIP-1α and RANTES. The expression of MCP-1 construct was similarly confirmed (data not shown).

IL-8 enhances T cell proliferation and Th1 isotype. The effects of various chemokines on vaccine-induced responses were analyzed individually. IL-8 is a potent chemotactic factor for neutrophil granulocytes and lymphocytes, and it is secreted by a variety of cell types, including T cells (15, 16). IL-8 binds to CXCR1, which is expressed on neutrophils, monocytes, and $CD8^+$ T cells (17, 18). The first immune parameter examined was the antigen-specific humoral response. Antisera from pCEnv and pCEnv+IL-8 immunized mice was collected and analyzed for specific antibody responses against HIV-1 gp120 protein by ELISA. Fig. 3 *A* shows the gp120-specific antibody titer from sera collected at weeks 0, 2, 4, and 6 after DNA immunization. At 1:128 dilution, sera from the groups immunized with pCEnv+IL-8 showed antibody response against gp120 protein, which was greater than that of the group immunized with pCEnv alone. A similar result was seen with the groups immunized with pCGag/pol (data not shown). Furthermore, the subclasses of gp120-specific IgGs induced by the coadministration with IL-8 genes were determined. It has been reported that production of IgG1 type is induced by Th2-type cytokines, whereas the IgG2a-type production is induced by Th1-type cytokines (19). The relative ratios of IgG1 to IgG2a (Th2 to Th1) are shown in Fig. 3 *B*. The pCEnv immunized group had a IgG1 to IgG2a ratio of 1.3. On the other hand, coinjection with pCEnv+IL-8 decreased the relative ratio to 0.9, indicating a shift to Th1-type response.

The effect of IL-8 expression on the T helper cell–proliferative response was also examined. As shown in Fig. 3, *C* and *D*, IL-8 coexpression with HIV-1 immunogens (pCEnv or pCGag/pol) resulted in a dramatic level of antigen-specific T helper cell–proliferative responses. The increase in proliferation was between four- and sixfold, a significant increase in antigen-specific responses. In addition, the effect of IL-8 coexpression on the induced CTL response was also investigated. As shown in Fig. 3 *E*, a background level of specific killing was observed from the control animals, whereas the animals immunized with pCEnv alone showed a small but consistent level of

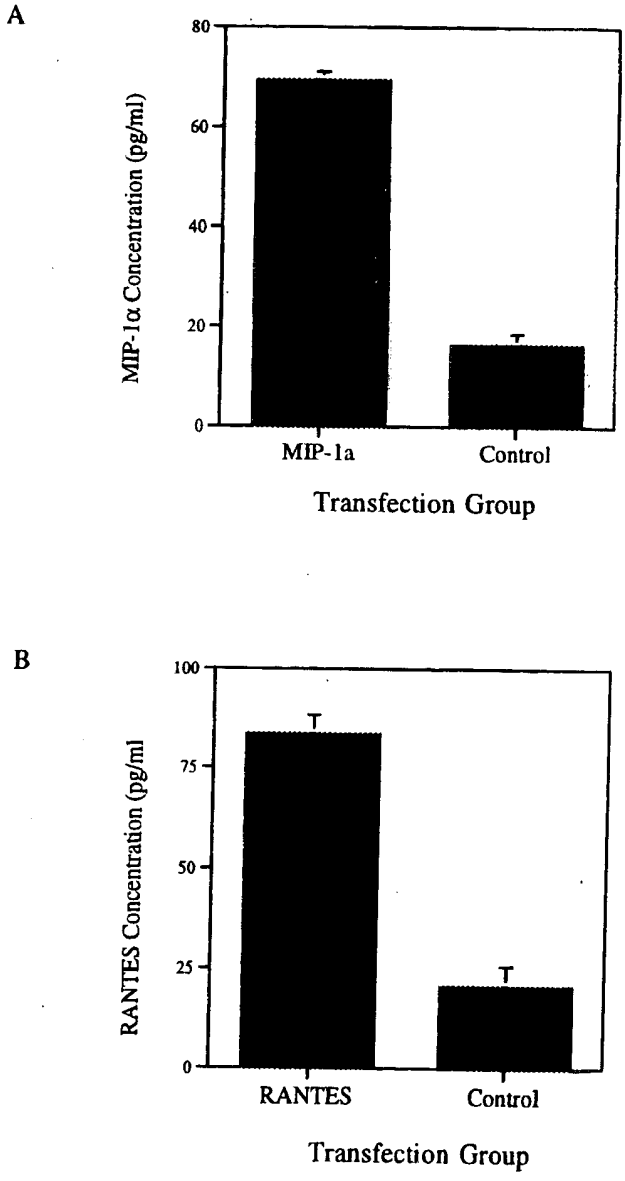

Figure 2. In vivo expression of the chemokine constructs. Mice were immunized intramuscularly with 50 μg of pCMIP-1α (*A*) or pCRANTES (*B*) or pCDNA3 (*Control*). The mice were killed, and their leg muscles were removed 3 d after injection. The muscle tissues were cultured in vitro for 72 h, and the supernatant were tested for the expression using the ELISA kits for MIP-1α (*A*) and RANTES (*B*).

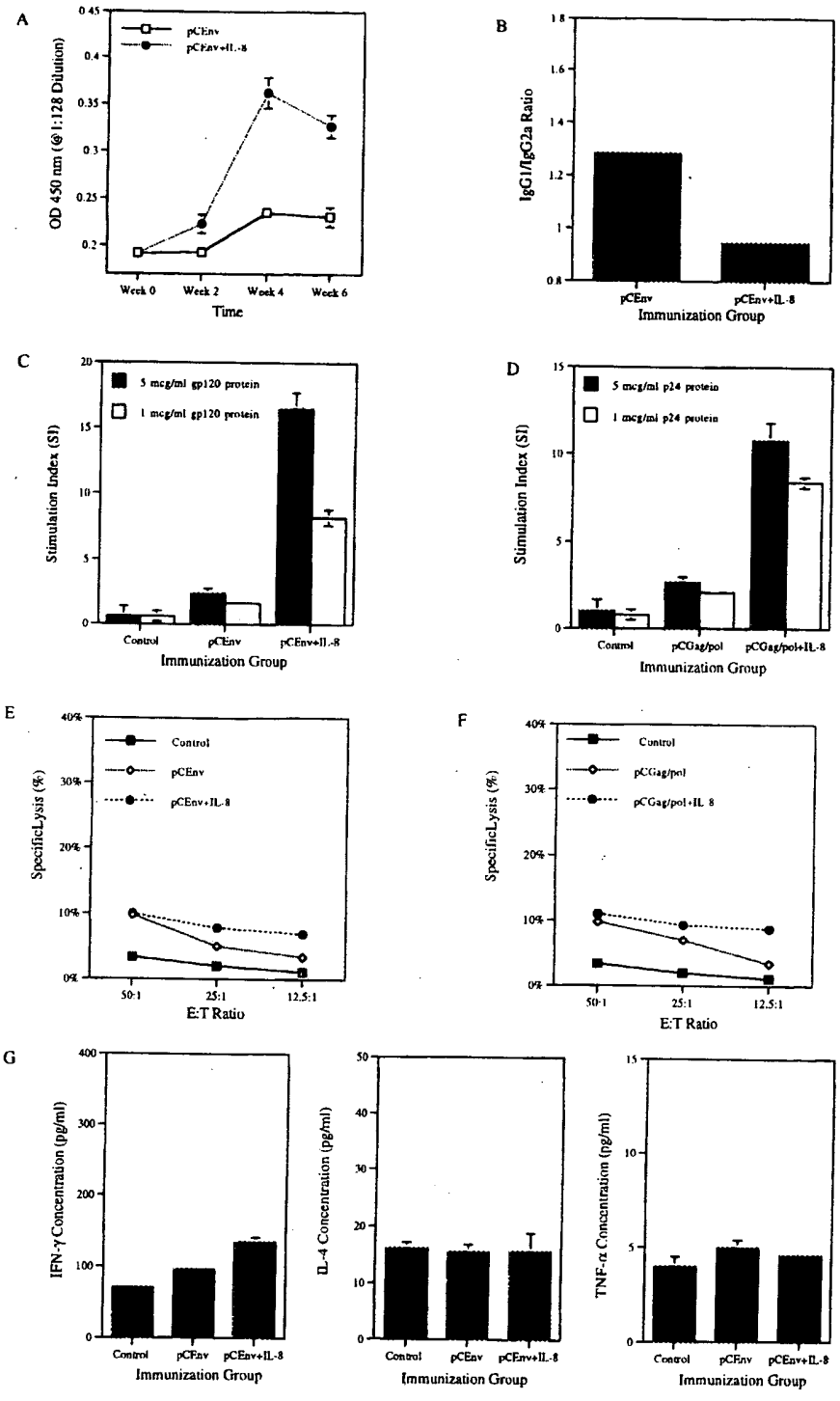

Figure 3. Antigen-specific immune responses after coimmunization with IL-8. Mice were coimmunized with 50 μg each of IL-8 and pCEnv expression cassettes intramuscularly at weeks 0 and 2. (*A*) HIV-1 envelope-specific antibody response after coimmunization. (*B*) HIV-1 envelope-specific IgG1/IgG2a ratio following coimmunization (at week 6). (*C*) HIV-1 envelope-specific Th cell–proliferative response after coimmunization. (*D*) HIV-1 gag/pol-specific Th cell–proliferative response following coimmunization. (*E*) HIV-1 envelope-specific CTL response after coimmunization. (*F*) HIV-1 gag/pol-specific CTL response after coimmunization. (*G*) Induction of cytokines IFN-γ, IL-4, and TNF-α in the supernatant of stimulated effector cells. These experiments have been repeated two times with similar results.

CTL response. IL-8 coadministration did not have any enhancement effect on the antigen-specific CTL response. Similar CTL results were observed from pCGag/pol+IL-8 coimmunization (Fig. 3 *F*).

Cytokines play a key role in directing and targeting immune cells during the development of the immune response. For instance, IFN-γ is intricately involved in the regulation of T cell-mediated cytotoxic immune responses (20), while IL-4 plays a dominant role in B cell–mediated immune responses (21). TNF-α is produced by activated macrophages and monocytes, neutrophils, activated lymphocytes, and natural killer (NK) cells, and has been suggested to play a pivotal role in regulating the synthesis of other proinflammatory cytokines (22). We analyzed supernatant from the effector cells stimulated in vitro for CTL assay and tested them for the release of cytokines IFN-γ, IL-4, and TNF-α. We found that IL-8 expression increased the level of IFN-γ only slightly, but it did not affect the levels of cytokines IL-4 and TNF-α (Fig. 3 *G*).

SDF-1α drives immune responses towards Th1-type immunity. We next examined the effects of SDF-1α codelivery on

vaccine induced immune responses. SDF-1α is a C-X-C chemokine, which binds to the CXCR4 (LESTR/fusin) receptor (4, 5). CXCR4 is expressed on a variety of leukocytes including monocytes/macrophages, neutrophils, B cells, and T cells (23). It is also a main coreceptor for entry of T cell–tropic HIV-1 strains (4, 5). Unlike IL-8, SDF-1α coinjection did not exhibit any effect on the level of antigen-specific humoral response (Fig. 4 *A*). Moreover, the relative ratios of IgG1 to IgG2a after the coadministration with pCEnv+SDF-1α were determined and are shown in Fig. 4 *B*. The pCEnv immunized group had a IgG1 to IgG2a ratio of 1.3. On the other hand, coinjection with pCEnv+SDF-1α decreased the relative ratio to 1.08, indicating a shift to Th1-type response. SDF-1α coinjection with HIV-1 immunogens (pCEnv or pCGag/pol) had small enhancement effect on the level of antigen-specific T helper cell–proliferative responses (Fig. 4, *C* and *D*). However, SDF-1α immunization had minimal effect on the antigen-specific CTL responses (Fig. 4, *E* and *F*) or on the induction of cytokines (Fig. 4 *G*).

MIP-1α is a strong expander of antibody response. MIP-1α is a C-C chemokine that binds to the receptors CCR1, CCR4, and CCR5. CCR1 is expressed on basophils and monocytes

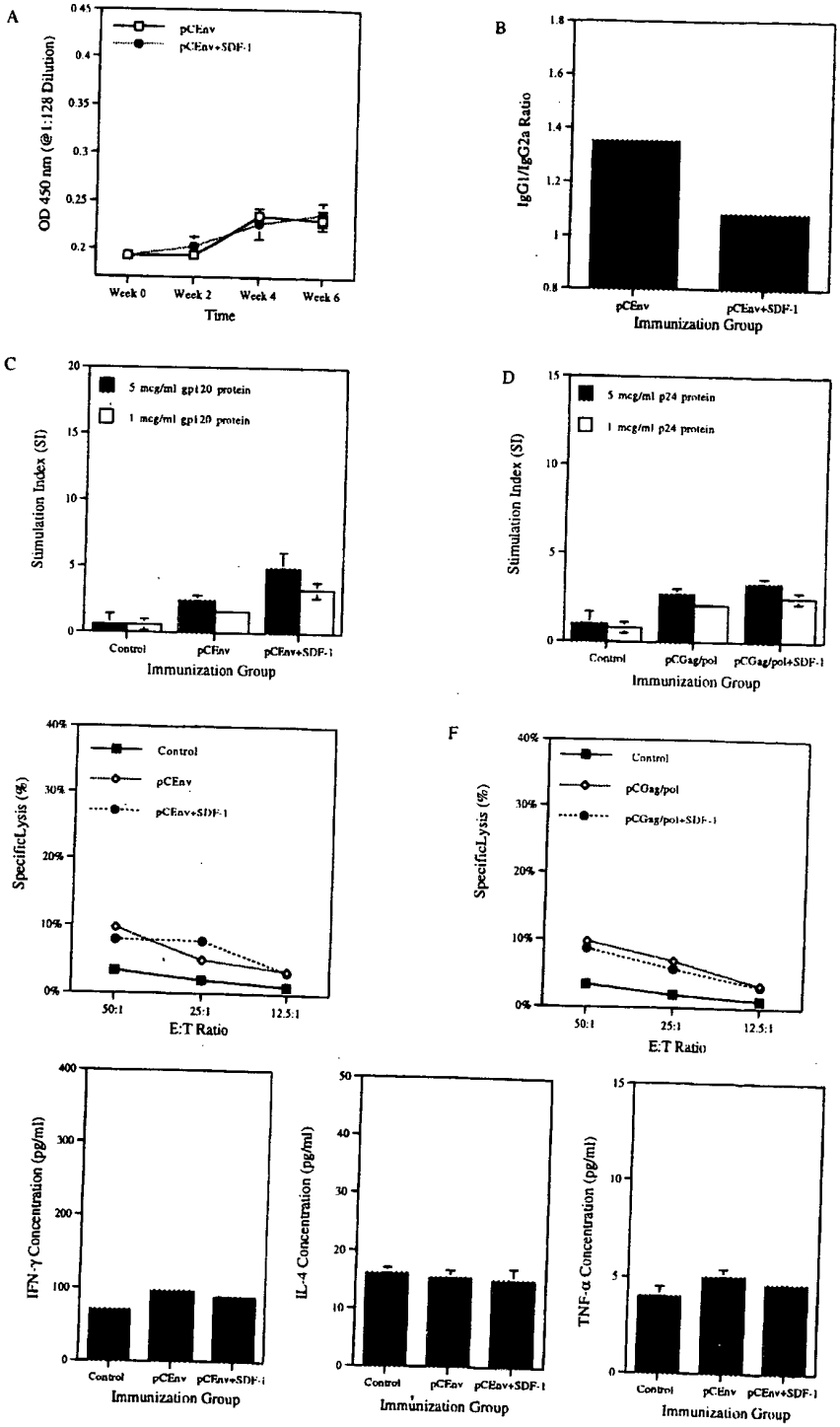

Figure 4. Antigen-specific immune responses after coimmunization with SDF-1α.

while CCR4 is expressed on monocytes, T cells, and B cells (1). CCR5, which is expressed on monocytes and macrophages, is a main coreceptor for entry of macrophage-tropic HIV-1 strains (6–8). MIP-1α coexpression exhibited a more drastic effect than IL-8 in the induction of antigen-specific humoral responses (Fig. 5 *A*). pCEnv+MIP-1α coimmunization resulted in a dramatic enhancement of envelope-specific antibody response. A similar result was seen with the groups immunized with pCGag/pol (data not shown). Moreover, the relative ratios of IgG1 to IgG2a after the coadministration with pCEnv+MIP-1α were determined and are shown in Fig. 5 *B*. The pCEnv immunized group had a IgG1 to IgG2a ratio of 1.3. On the other hand, coinjection with pCEnv+MIP-1α increased the relative ratio to 1.7, indicating a shift to a more Th2-type response. MIP-1α coexpression with HIV-1 immunogens (pCEnv or pCGag/pol) resulted in enhancement of antigen-specific T helper cell–proliferative responses (Fig. 5, *C* and *D*). In contrast, MIP-1α immunization had minimal effect on the antigen-specific CTL responses (Fig. 5, *E* and *F*) or the induction of cytokines (Fig. 5 *G*).

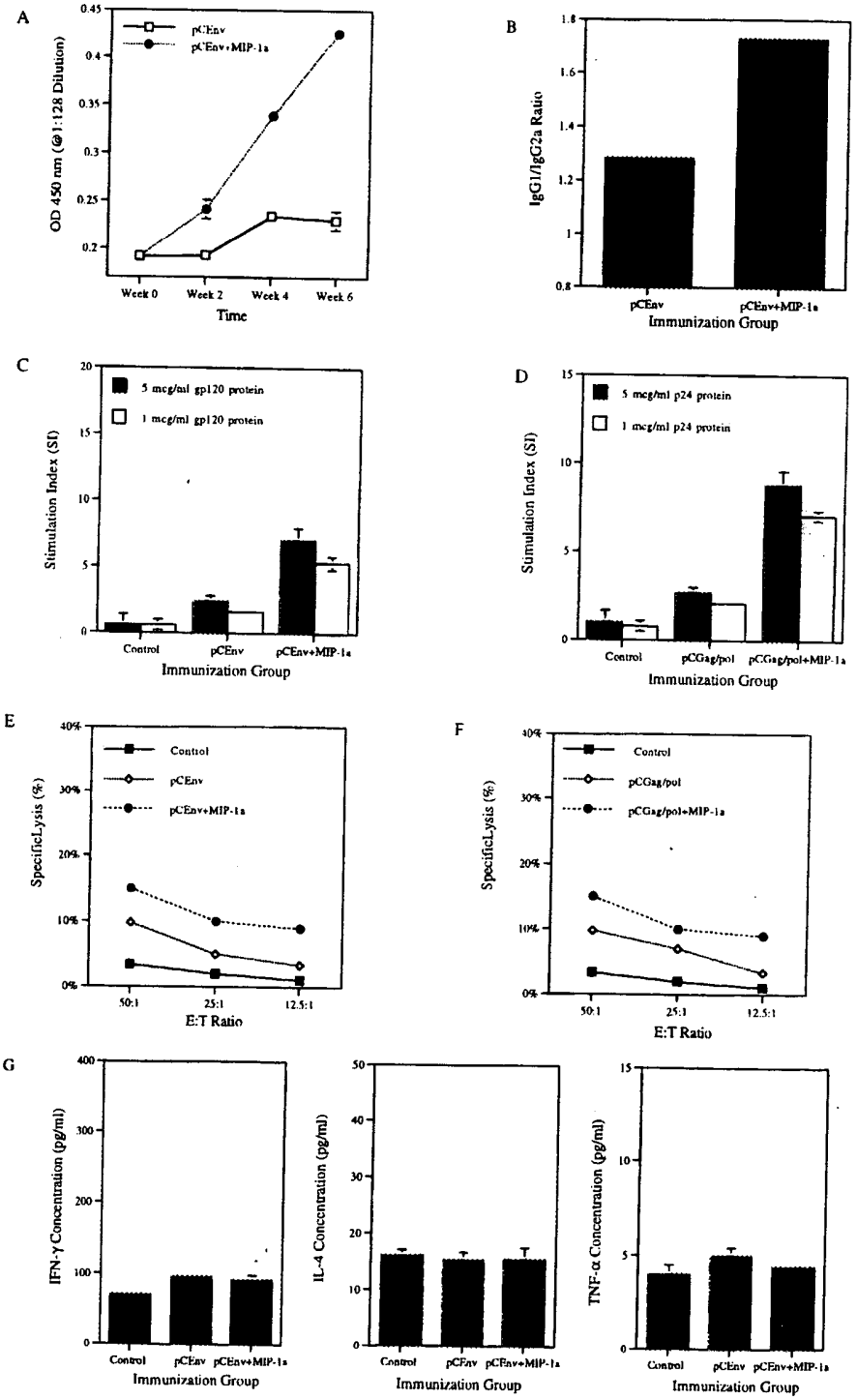

Figure 5. Antigen-specific immune responses after coimmunization with MIP-1α.

RANTES expands Th1 as well as CTL responses. We next examined the effects of RANTES codelivery on vaccine-induced immune responses. RANTES binds to the CCR1, CCR3, CCR4, and CCR5 receptors. CCR3 is expressed on eosinophils and monocytes. CCR3 is also a coreceptor for entry of macrophage-tropic HIV-1 strains, but it is less prominent than CCR5 (24, 25). Unlike IL-8 and MIP-1α, coexpression of RANTES with pCEnv did not significantly enhance HIV-1 envelope-specific antibody response (Fig. 6 *A*). In addition, pCEnv+RANTES coimmunization did not have any effect on the IgG1/IgG2a ratio when compared with the group immunized with pCEnv alone (Fig. 6 *B*). In contrast to the antibody responses, RANTES covaccination with HIV-1 immunogens (pCEnv or pCGag/pol) resulted in significant augmentation of antigen-specific T helper cell–proliferative responses (Fig. 6, *C* and *D*). Furthermore, twofold higher level expression of Th1 cytokines IFN-γ and TNF-α was observed from the group coadministered with pCEnv+RANTES (Fig. 6 *G*). Unlike coinjection with pCEnv+IL-8 or pCEnv+MIP-1α, which resulted in a minimal effect in CTL activity, a more dramatic increase in the specific killing of targets infected with vaccinia (vMN462) expressing HIV-1 envelope was observed

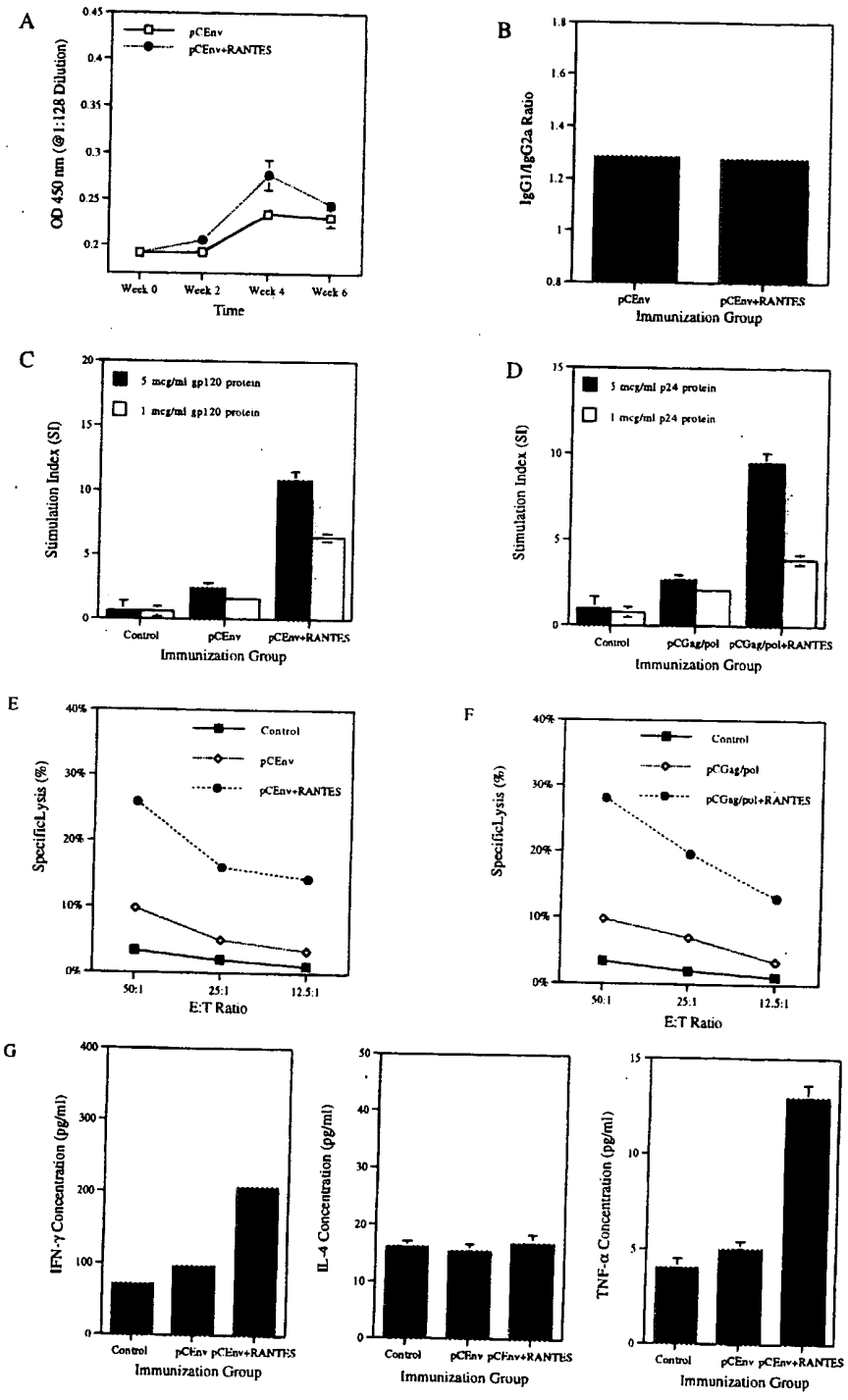

Figure 6. Antigen-specific immune responses after coimmunization with RANTES.

after coinjection with pCEnv+RANTES (Fig. 6 *E*). Almost 30% specific lysis of target cells was observed after coinjection with pCEnv+RANTES at a 50:1 E/T ratio. Similarly, the mice immunized with pCGag/pol+RANTES resulted in a significant enhancement of antigen-specific CTL lysis of targets infected with vaccinia (vVK1) expressing HIV-1 gag/pol (Fig. 6 *F*).

MCP-1 is a potent expander of CTL responses. Adjuvant properties of MCP-1 cDNA were next observed. MCP-1 is a C-C chemokine that binds to the CCR2 receptor. Similar to CCR3 and CCR5, CCR2 is a coreceptor for entry of macrophage-tropic HIV-1 strains, but it is less prominent than CCR5 (25). MCP-1 appeared to have a minimal effect on the specific antibody-binding profile induced by pCEnv immunization. Moreover, MCP-1 coexpression with HIV-1 immunogens (pCEnv or pCGag/pol) had positive but relatively minor (twofold) enhancement of antigen-specific T helper cell–proliferative responses (Fig. 7 *A*). Furthermore, the relative ratios of IgG1 to IgG2a after the coadministration with pCEnv+MCP-1

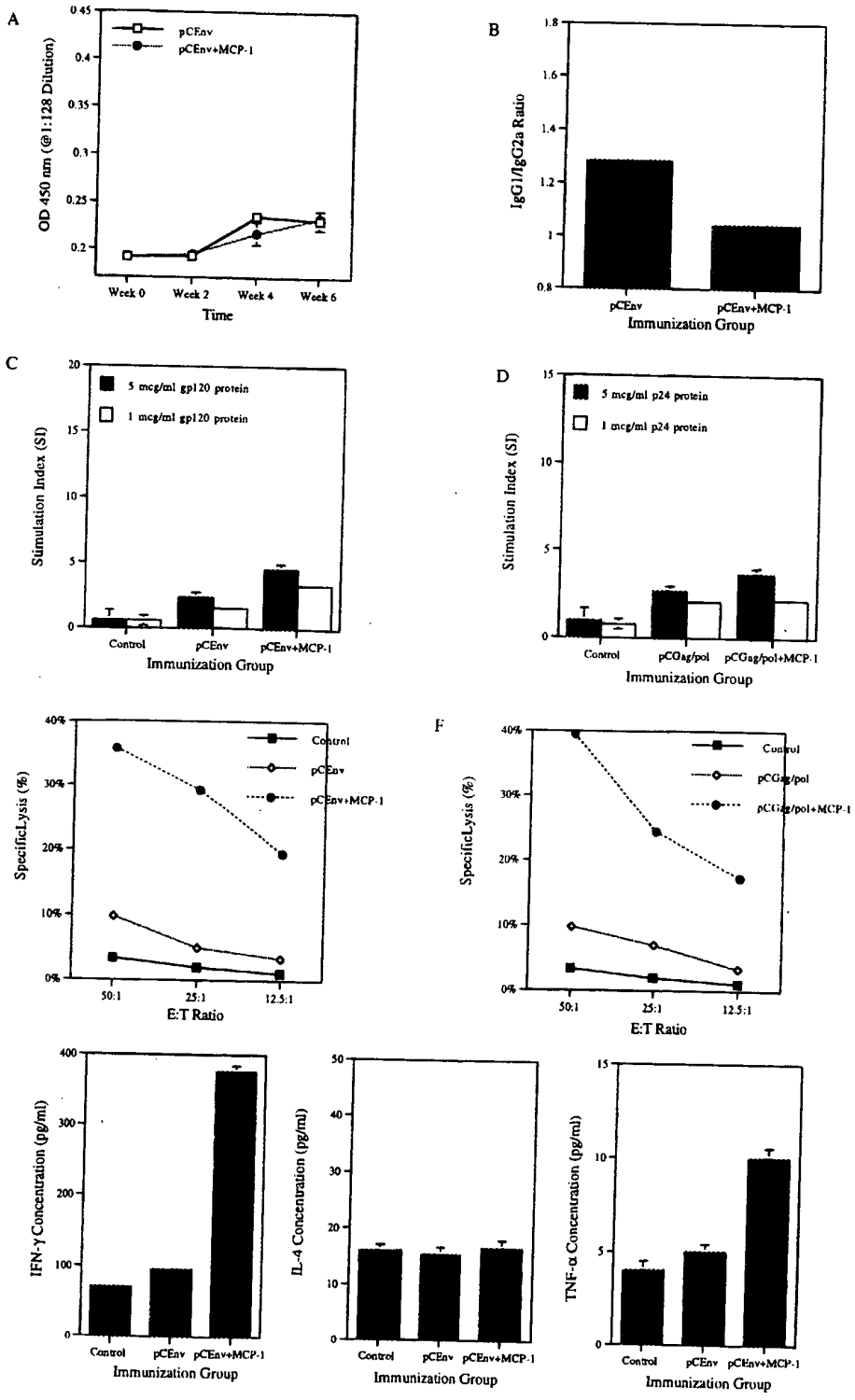

Figure 7. Antigen-specific immune responses after coimmunization with MCP-1.

were determined and are shown in Fig. 7 *B*. The pCEnv immunized group had a IgG1 to IgG2a ratio of 1.3. On the other hand, coinjection with pCEnv+MCP-1 decreased the relative ratio to 1.0, indicating a shift to Th1-type response. A more dramatic increase in the specific killing was observed after coinjection with pCEnv+MCP-1 (Figure 7 *E*). > 36% specific lysis of target cells was observed after coinjection with pCEnv+MCP-1 at a 50:1 E/T ratio. Similarly, the mice immunized with pCGag/pol+MCP-1 resulted in a significant enhancement of antigen-specific CTL lysis of HIV-1 gag/pol expressing targets (Fig. 7 *F*). As shown in Fig. 7 *G*, the level of IFN-γ release by mice immunized with pCEnv+MCP-1 was significantly greater than those of the pCEnv immunized or the control groups. Again, the level of IL-4 released from all groups was similar. Moreover, the level of TNF-α release by pCEnv+MCP-1 immunized group was significantly greater than those of the pCEnv immunized or the control groups. These cytokine release data support the CTL above results implicating MCP-1 as an activator of $CD8^+$ CTL.

Determination of CD8 restriction in CTL response. To determine whether the increases in CTL response via coexpression of MCP-1 and RANTES were restricted to $CD8^+$ T cells, CTL assays were performed using a HIV-1 envelope peptide (RIHIGPGRAFYTTKN) pulsed cells as targets. This peptide has been shown to be a specific epitope for MHC class I–restricted CTL in Balb/c mice (12). Mice received two immunizations of 50 μg of each DNA construct separated by 2 wk, and their spleens were harvested 1 wk after the second immunization. The CTL assay was performed on the splenocytes after in vitro stimulation with envelope-specific peptides as previously described (10, 11). We observed a significant enhancement of CTL response after coinjection (Fig. 8) with MCP-1 and RANTES at 35 and 26% specific killing at an E/T ratio of 50:1, respectively. We verified this observation by measuring CTL activity after the removal of $CD8^+$ T cells from the effector cell population by complement lysis (10). As shown in Fig. 9, the removal of $CD8^+$ T cells resulted in the suppression of antigen-specific CTL enhancement observed after coinjections with MCP-1 and RANTES. These results indicate that the enhancement of cytolytic activity was antigen specific and $CD8^+$ T cell dependent.

Enhancement of chemokine expression. It was important to determine the effects, if any, of these specific chemokine adjuvants on chemokine production itself. We examined the expression of chemokines MIP-1α, RANTES, and MCP-1 by stimulated cells collected from immunized animals. Chemokine coinjection modulated chemokine production in chemokine-specific patterns. Coimmunization with chemokine cDNA cassettes resulted in increased expression of chemokines in an antigen specific manner. As shown in Fig. 10, MIP-1α, RANTES, or MCP-1 expression was enhanced dramatically by coimmunization with pCEnv+MIP-1α pCEnv+RANTES, pCEnv+MCP-1, respectively.

Discussion

The initiation of immune or inflammatory reactions is a complex process involving a tightly coordinated expression of cel-

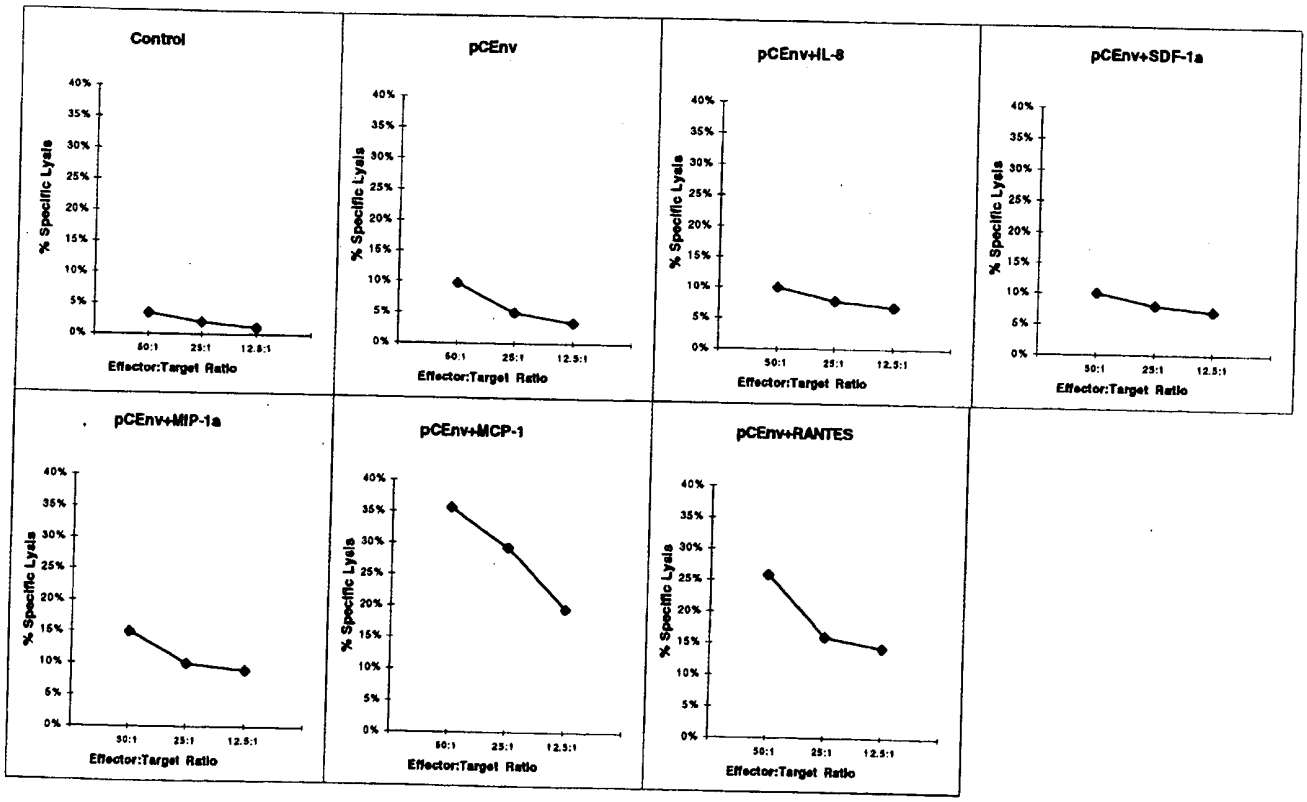

Figure 8. Determination of MHC class I–restricted CTLs. CTL assay was performed using effector cells and prepared as described, and target cells prepared with envelope-specific peptide (RIHIGPGRAFYTTKN), that has been reported to be MHC class I-restricted in Balb/c mice. These experiments have been repeated two times with similar results.

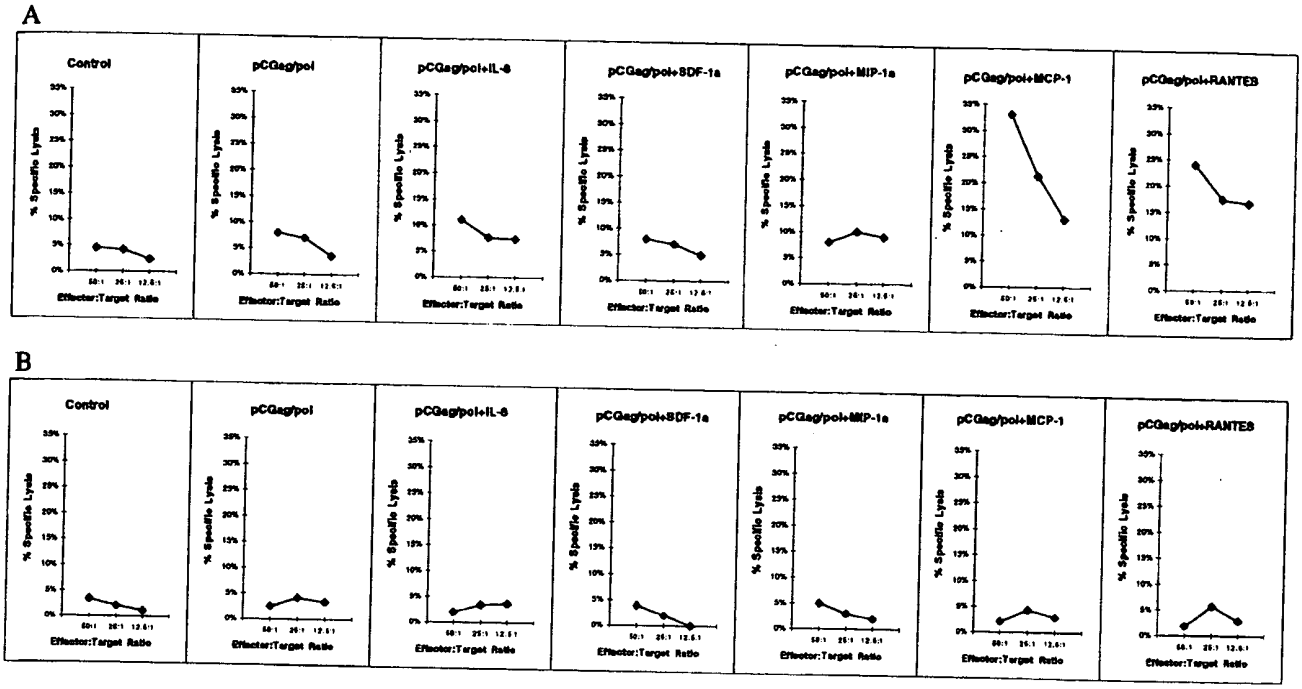

Figure 9. Determination of $CD8^+$-dependent CTLs. A CTL assay was performed with the removal of $CD8^+$ T cells by complement lysis. Effector cells were prepared as described with the presence of $CD8^+$ T cells (*A*) and the removal of $CD8^+$ T cells (*B*) using vaccinia-infected target cells. These experiments have been repeated two times with similar results.

lular adhesion molecules, cytokines, and chemokines. The chemokines are especially important in the molecular regulation of trafficking of leukocytes from the lymph and blood vessels to the peripheral sites of host defense. The superfamily of chemokines consists of an array of over 30 related proteins (1). In addition to their functions in inflammatory and immune responses, some chemokines play a critical role in the transmission and progression of HIV-1 and 2 viruses responsible for AIDS. Recent studies have identified that the coreceptors required for the fusion of the T cell–tropic and macrophage-tropic viruses with their target cells to be CXCR4 and CCR5, respectively (4–8).

To elucidate the specific roles of these chemokines in immune induction and modulation, we used the codelivery of chemokine DNA expression cassettes as an antigen delivery model. DNA coimmunization is an appropriate model to investigate the in vivo functions of chemokines because DNA vaccines induce both humoral and cellular immune responses via both the MHC class I and II pathways (10, 26–29). Furthermore, we and others have shown that antigen-specific immune responses to DNA vaccines can be modulated by the coinjection of costimulatory molecule and cytokine genes with DNA immunogen cassettes (10, 11, 13, 30–34). Thus, we cloned and coimmunized chemokine expression vectors with HIV-1 DNA immunogens and examined the effects of chemokine expression on immune activation. We observed that the α-chemokine IL-8 and SDF-1α as well as the β-chemokines MIP-1α, RANTES, and MCP-1 had specific, identifiable roles in the activation of antigen-specific immune responses.

For instance, IL-8 is a chemotactic factor for neutrophils, inducing them to leave the bloodstream and migrate into the surrounding tissues. We observed that IL-8 was a strong in-

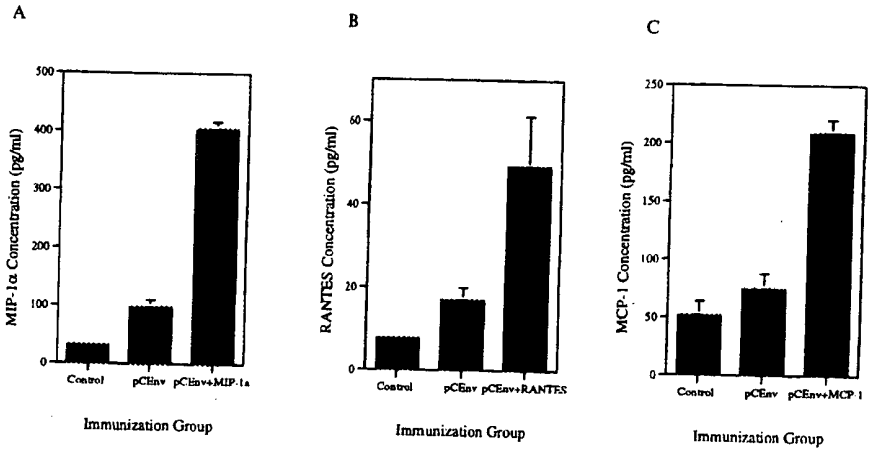

Figure 10. Expression of chemokines by stimulated effector cells. Supernatants from effectors stimulated for CTL assay were collected at day 6 and tested for cytokine profile using specific chemokine ELISA kits for MIP-1α (*A*), RANTES (*B*), and MCP-1 (*C*) as described. These experiments have been repeated two times with similar results.

ducer of $CD4^+$ T cells, resulting in strong T helper–proliferative responses as well as increasing antibody responses. IL-8 coexpression also modulated the shift of immune responses to Th1-type, indicated by the reduction of IgG1 to IgG2a ratio and enhanced expression of IFN-γ. On the other hand, IL-8 coadministration did not seem to have noticeable effect on $CD8^+$ T cells, since it did not have any enhancement effect on the CTL response.

SDF-1 interestingly binds directly to CXCR4 receptors, which are expressed on both $CD4^+$ as well as $CD8^+$ T cells (4, 5). As this chemokine could directly interact with antigen-stimulated T cells, it was somewhat surprising to note its lack of effect on either T helper or CTL responses. The major effect observed was a shift in isotype towards a Th1 type, based on the IgG subtype ratios.

It has been reported that MIP-1α can chemoattract and degranulate eosinophils (35). MIP-1α also induces histamine release from basophils and mast cells and is a chemotactic factor for basophils and B cells (36, 37). These studies support our observation that MIP-1α had the greatest effect on antibody responses. In addition, MIP-1α was also a strong inducer of T helper–proliferative responses. MIP-1α coexpression also modulated the shift of immune responses to Th2 type, indicated by the increase of IgG1 to IgG2a ratio. In contrast, MIP-1α coimmunization had minimal effect on the $CD8^+$ T cell response.

Unlike the effects of MIP-1α, RANTES coimmunization had minimal effect on antibody responses. RANTES is a monocyte chemoattractant. In addition, RANTES can chemoattract unstimulated $CD4^+/CD45RO^+$ memory T cells and stimulated $CD4^+$ and $CD8^+$ T cells (38–40). This ability of RANTES to chemoattract $CD4^+$ and $CD8^+$ T cells to the site of DNA immunization may have been important in inducing T helper–proliferative responses and CTL responses. The enhanced activation of Th1 responses was supported by the increased expression of Th1 cytokines IFN-γ and TNF-α. The high level of CTL responses induced by RANTES expression was determined to be class I–restricted and $CD8^+$ T cell dependent.

As a potent chemotactic factor for monocytes, MCP-1 is thought to be an important chemokine in chronic inflammatory disease (41). MCP-1 induces monocytes to migrate from the bloodstream to become tissue macrophages. MCP-1 was also found to chemoattract T lymphocytes of the activated memory subset (42). Among all chemokines examined, MCP-1 is the most potent activator of $CD8^+$ CTLs. The enhancement of CTL responses induced by MCP-1 expression was determined to be class I–restricted and $CD8^+$ T cell dependent. The enhanced CTL results are supported by increased expression of Th1 cytokines IFN-γ and TNF-α and the reduction of IgG1 to IgG2a ratio. Unlike RANTES, MCP-1 had positive, but moderate effect on the T helper cell–proliferative responses. Like RANTES, MCP-1 coadministration had no effect on antibody responses. This comparison highlights that while the induction of humoral, T helper, and T cytotoxic responses are coordinately regulated and integrated, they can be modulated independently of each other, depending on the specific environment in which the response is triggered.

In addition to their direct effects on immune responses, coexpression of chemokine genes resulted in their increased expression in autocrine manner. For instance, we observed that MIP-1α expression could be enhanced dramatically by coimmunization with pCEnv+MIP-1α over the level expressed by pCEnv immunization alone. Similar increases in RANTES were observed from RANTES codelivery. These results imply that these chemokines not only have a direct role in modulating immune responses, but they also amplify their effects by inducing more production of chemokines in autocrine manner. This feature could be exploited for developing immunogens that drive chemokine production as well as humoral responses to cooperatively block lentiviral infection.

An important observation was the role chemokines RANTES and MCP-1 play in inducing TNF-α expression. TNF-α is produced by activated macrophages and monocytes, neutrophils, activated lymphocytes, and NK cells (43). TNF-α is also implicated in septic shock after infection by Gram-negative bacteria (44) and in rheumatoid arthritis (45). Furthermore, TNF-α plays a pivotal role in regulating the synthesis of other proinflammatory cytokines (22). Given TNF-α's critical roles in various ailments, there have been major efforts in reducing the level of TNF-α in vivo as potential treatment for conditions such as rheumatoid arthritis. In our experiments, we observed that coexpression of RANTES or MCP-1 resulted in the enhanced expression of TNF-α. These results suggest that inhibiting RANTES and MCP-1 should be examined as a strategy to downregulate TNF-α expression in vivo.

It is of interest that Th1 vs. Th2 phenotype appears to segregate independently of other immune functions. IL-8 boosted humoral responses but drives those responses towards a Th1 phenotype, cutting the IgG1/IgG2a ratio by almost 50% (Fig. 3 *B*). MIP-1α, perhaps the most prolific driver of serology, skewed the IgG1/IgG2a ratio dramatically towards a Th2 response (Fig. 5 *B*). It is clear that this manipulation can allow for induction of primary antigen-specific immune responses skewed towards a desired phenotype as well as immunoglobulin isotype independently of each other. Furthermore, the induction of cellular vs. higher humoral responses appeared to be relatively polarized immune functions. Those chemokines with the most dramatic effect on humoral responses, IL-8 and MIP-1α, exhibited little effect on CTL responses whereas those that mediated the most dramatic effects on CTL responses, RANTES and MCP-1, had minimal effects on serology. The same CTL driving chemokines RANTES and MCP-1 both stimulated IFN-γ and TNF-α, while the humoral responders had minimal effects on these important cytokines.

IL-8, SDF-1α, MIP-1α, MIP-1β, and RANTES are products of $CD8^+$ effector cells as well as other cells. Collectively, these studies demonstrate that these chemokines can function in a manner more similar to traditional Th1 or Th2 cytokines in driving and expanding immunity. For example, while MIP-1α can drive humoral responses, RANTES can drive CTLs. This finding suggests that $CD8^+$ T cells, like $CD4^+$ T cells, can play an important role, and perhaps a decisive role, in immune expansion (Fig. 11). However, this expansion generally would take place at the site of high-antigen density during effector cell function rather than in the lymphoid organs (Fig. 11). For example, as a part of active $CD8^+$ effector cell function at the site of viral clearance, the local production of specific chemokines would drive expansion of humoral and/or cellular immunity until $CD8^+$ CTLs eliminated their targets and ceased being activated. Since $CD8^+$ T cells are ultimately responsible for viral clearance, it is advantageous that they would control immune responses at the front line battle site as they have the specific machinery to determine when the invading pathogen

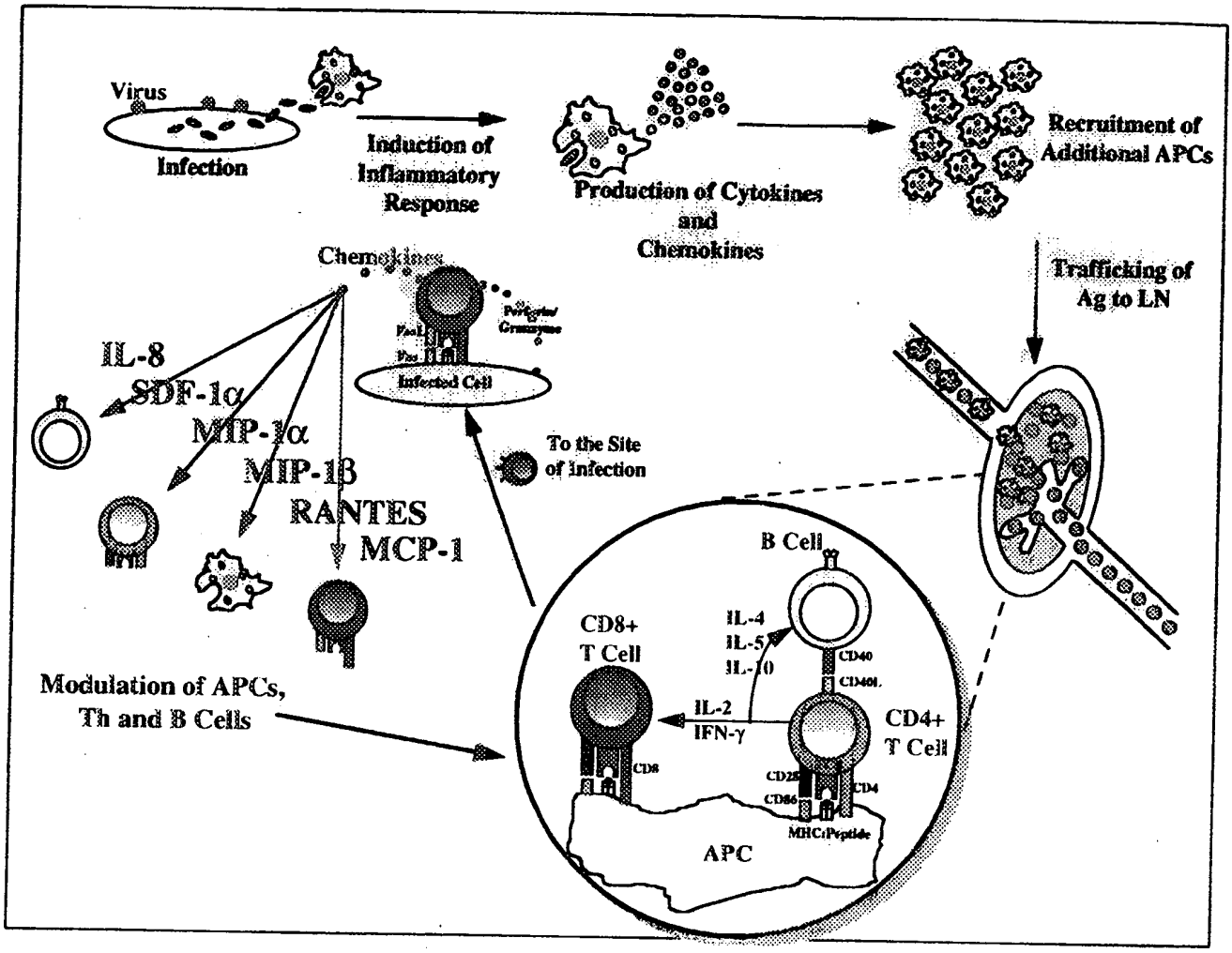

Figure 11. A summary of immune expansion mediated by chemokines and CD8⁺ T cells. As a part of active effector cell function in the periphery, activated CD8⁺ CTLs produce specific chemokines which drive immune expansion.

has been cleared. The site of immune expansion would distinguish this regulatory role from the role of CD4⁺ T cells that require APCs and class II presentation to assert their role in immune regulation. In contrast, peripheral CTL action requires only class I presentation that can be achieved by non-APCs. How and if CD8 effector cells segregated into "Th1 versus Th2 chemokine" producing cells is a subject for future investigation.

These results outline a key regulatory role for CD8⁺ effector cells in the expansion phase of an antigen-specific immune response in the periphery. Better understanding of the mechanism of immune expansion could have important implication for the design of vaccines and immune therapies.

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EXHIBIT B

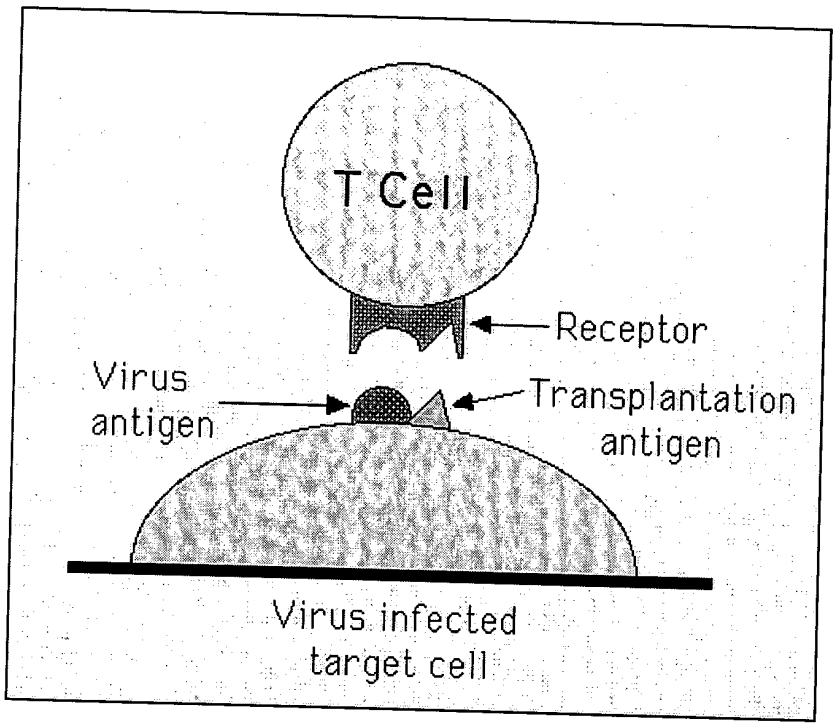

Figure legend: The figure describes how a killer T lymphocyte must recognize both the virus antigen and the self histocompatibility antigen molecule in order to kill a virus-infected target cell. The figure is a modification of the figure published by Zinkernagel and Doherty already 1974 (in Nature 251, p 547).

Arch Pathol Lab Med 1998 Jun;122(6):523-33

Human immunodeficiency virus-2 infection in baboons is an animal model for human immunodeficiency virus pathogenesis in humans.

Locher CP, Barnett SW, Herndier BG, Blackbourn DJ, Reyes-Teran G, Murthy KK, Brasky KM, Hubbard GB, Reinhart TA, Haase AT, Levy JA.

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OBJECTIVE: To assess disease progression in baboons (Papio cynocephalus) that were infected with two human immunodeficiency virus-2 (HIV-2) isolates. METHODS: Eight baboons were inoculated intravenously with either HIV-2UC2 or HIV-2UC14 and were followed for a 2- to 7-year period of observation. RESULTS: Six of 8 baboons showed lymphadenopathy and other signs of HIV-related disease, 3 of 8 baboons had an acute phase CD4+ T-cell decline, and 2 of 5 baboons infected with the HIV-2UC2 isolate progressed to an acquired immunodeficiency syndrome-like disease. Human immunodeficiency virus-2-specific pathology in lymphatic tissues included follicular lysis, vascular proliferation, and lymphoid depletion. Both neutralizing antibodies and a CD8+ T-cell antiviral response were associated with resistance to disease. CONCLUSIONS: Disease progression and the development of acquired immunodeficiency syndrome in HIV-2-infected baboons have similarities to human HIV infections.

PMID: 9625420 [PubMed - indexed for MEDLINE]

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LWW ONLINE

Infection of baboons with a simian immunodeficiency virus/HIV-1 chimeric virus constructed with an HIV-1 Thai subtype E envelope.

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OBJECTIVE: To construct an infectious chimeric simian immunodeficiency virus/HIV-1 (SHIV) with the envelope of a Thai subtype E HIV-1 strain for use in a non-human primate model. METHODS: A novel SHIV genome was derived using the sequences of the ectodomain of the envelope gene from the Thai subtype E strain, HIV-1(9466). This SHIV (SHIV9466.33) was recovered by cocultivation from human peripheral blood mononuclear cells (PBMC) after transfection of human rhabdosarcoma cells. Rhesus macaque and baboon PBMC were screened in vitro for susceptibility to infection with SHIV9466.33. After successful infection of baboon PBMC, four animals were inoculated intravenously with SHIV9466.33 and monitored for plasma viral RNA, virus isolation from the PBMC, seroconversion, T-cell subsets, and signs of disease. RESULTS: SHIV9466.33 was able to infect PBMC from 12 out of 14 baboons. All four of the baboons selected for in vivo inoculation became infected. Peak plasma viral RNA levels of 8000 to 700,000 RNA copies/ml were measured at 2 weeks post-inoculation. Virus was isolated from the PBMC of all four baboons during acute infection, and all seroconverted. Although transient declines in CD4+ T-cells were observed during early infection, CD4+ levels remained within normal ranges thereafter. In contrast, in vitro cultures of PBMC of four rhesus macaques were not susceptible to infection with SHIV9466.33. CONCLUSION: SHIV9466.33 containing an HIV-1 subtype E envelope displayed tropism for baboon PBMC but not for rhesus macaque PBMC. This chimeric virus established infection and induced antiviral antibodies in baboons inoculated by the intravenous route with cell-free virus. Thus, infection of baboons with SHIV9466.33 will serve as an important animal model for future studies of HIV-1 vaccine efficacy.

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Epidemiology of herpesvirus papio infection in a large captive baboon colony: similarities to Epstein-Barr virus infection in humans.

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The epidemiology of herpesvirus papio, a lymphocryptovirus similar to Epstein-Barr virus (EBV), was studied in a captive colony of >1900 baboons. Herpesvirus papio IgG antibody titers were measured by IFA. In total, 438 specimens from 296 baboons were assessed, including 116 serial specimens from 52 juveniles and 6 infants studied monthly for 1 year following birth and at age 18 months. Maternally derived antibody reached a nadir at 4 months of age. About 75% of animals at 12 months of age and >95% of animals after age 24 months demonstrated serologic evidence of herpesvirus papio infection. After age 3 years, the geometric mean titer was 1:60-75. The epidemiology of herpesvirus papio infection in baboons closely parallels that of EBV infection in humans. An animal model of lymphocryptovirus infection will facilitate investigations of human lymphocryptovirus biology.

PMID: 10762578 [PubMed - indexed for MEDLINE]

Transplantation 2001 Nov 15;72(9):1541-8

In vitro recognition and impairment of pig islet cells by baboon immune cells: similarity to human cellular reactions.

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BACKGROUND: Grafting pig islets into patients with type 1 diabetes requires control of the strong cellular xenogeneic rejection. This in vitro study compared the cellular reaction of baboons and humans to pig islet cells (PICs) to confirm the validity of using these animals for further in vivo preclinical trials. METHODS: Baboon or human peripheral blood mononuclear cells (PBMCs) or subsets were co-incubated with PICs from specific pathogen-free adult pigs for 7 days to determine the mechanisms and intensity of PBMC proliferation. Interleukin (IL) 10 and interferon (IFN) gamma secretion were assessed by enzyme-linked immunosorbent assay. Because proliferation was not indicative of aggression, a test based on perifusion analysis of the alteration of basal and stimulated insulin releases from PIC incubated with different baboon and human cells was developed. RESULTS: Baboon PBMCs strongly proliferated in response to PICs (stimulation index [SI]=24.8+/-6.9 [n=8] vs. 23.9+/-3.4 [n=34] for human PBMCs), showing considerable variation in intensity among animals (2.3<SI<63) and humans (1.8<SI<97). PBMC proliferation was inhibited in baboons and humans by anti-CD4 (% inhibition of SI: 71+/-10% and 75+/-7%, respectively) and anti-DR (75+/-35% and 80+/-6%) monoclonal antibodies (MoAbs) or by depletion of MHC class II+ cells (99+/-1% and 90+/-6%). Blocking by anti-CD8 or anti-CD16 MoAbs was weaker and variable among both animals and humans. IL-10 production by baboon and human PBMCs in response to PICs increased more than IFN-gamma production after 2 days of coculture, but the IL-10/IFN-gamma ratio was inverted after 5 days of coculture. After 7 days (and even after only 2 days) of coculture with baboon (n=8) or human (n=18) PBMCs, basal and glucose-stimulated insulin secretions from PICs were almost completely abolished (P<0.0001). The drop in insulin release could have mainly resulted from lysis of PICs, because the number of PICs decreased by 78% after 7 days of co-incubation with PBMCs. A decrease of insulin release by PBMCs was reproduced with plastic-adherent cells and was abolished by depletion of MHC class II+ cells or by addition of 100 microg/ml gadolinium (which inhibits macrophages), but not by cyclosporine. In baboons, as in humans, insulin release was also decreased after coculture of PICs with enriched T lymphocytes remixed with antigen-presenting cells (APCs). CONCLUSIONS: This study provides the first data on in vitro comparison of baboon and human cell-mediated recognition and impairment of PICs. Proliferation of PBMCs against PICs involves mainly CD4 T cells, with indirect recognition mediated by baboon or human MHC class II+ APCs. The Th2/Th1 profile of cytokines secreted in response to PICs was similar in baboon and human PBMCs. The model based on alteration of insulin secretion indicates that PIC impairment by whole mononuclear cells was strong and rapid and that a crucial role was played by MHC class II+ and plastic-adherent cells. Two mechanisms appear to be responsible for the role of these cells: (1) early and strong direct effect, which is potentially involved in vivo in primary nonfunction of islets aggressed by monocytes and macrophages; and (2) presentation of PIC xenoantigens, which leads to impairment by T lymphocytes possibly involved in in vivo-specific cellular rejection. The mechanisms and intensity of baboon cellular reactions to PICs in vitro were similar to those observed in humans, which suggests that the baboon is a suitable model for the study of cellular mechanisms during preclinical trials of pig islet xenografts.

PMID: 11707743 [PubMed - indexed for MEDLINE]

1: Dev Biol Stand 1983;53:257-61

Infection of baboons ("Papio cynocephalus") with rotavirus (SA11).

Kalter SS, Heberling RL, Rodriguez AR, Lester TL.

A recent survey of nonhuman primate sera indicated that antibody to a rotavirus (SA11) was prevalent among a wide spectrum of animals. Both New and Old World species were found with antibody, many with surprisingly high titers (1:320). Whether or not infection per se was due to SA11 or an antigenically closely related agent could not be determined by that study; however, it is apparent from this study as well as from a survey of the literature that natural as well as experimental infection of nonhuman primates occurs. Recognizing that there is a need for an animal model for the study of viruses associated with human diarrhea, a preliminary investigation attempting to ascertain the susceptibility of the baboon (Papio cynocephalus) to a rotavirus was undertaken. Inoculation of four newborn baboons with SA11 resulted in diarrhea within 24 to 48 hours in all animals. One animal died 10 days postinoculation following severe diarrhea and dehydration. Virus was isolated as well as seen by electron microscopy in the stools of all four animals.

Publication Types:

- Review

PMID: 6307781 [PubMed - indexed for MEDLINE]

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Baboons as an animal model for human immunodeficiency virus pathogenesis and vaccine development.

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Baboons (Papio cynocephalus) provide a valuable animal model for the study of human immunodeficiency virus (HIV) pathogenesis because HIV-2 infection of baboons causes a chronic viral disease that progresses over several years before clinical signs of acquired immunodeficiency syndrome (AIDS) appear. Since HIV-2-infected baboons develop a chronic viral infection, insights into the immuno-biology of viral latency, clinical stages of disease, virus infection of lymphatic tissue and HIV transmission can be gained using this animal model. The development of an AIDS-like disease in baboons is viral isolate and baboon subspecies dependent. Thus, viral virulence factors and host resistance can be studied as well as the mechanisms of innate and acquired immunity. The control of virus infection is dependent upon cytotoxic and non-cytotoxic antiviral activity of CD8+ T cells. In this regard, some of the HIV-2-infected baboons develop potent antiviral cellular immune responses that have a similar magnitude to that found in HIV-1-infected long-term survivors (or non-progressors). In our laboratory, baboons have been used to study DNA vaccine strategies using new cationic liposome formulations and granulocyte macrophage-colony stimulating factor and B7-2 as genetic adjuvants. The results demonstrate the value of using baboons as an animal model of AIDS pathogenesis and vaccine development.

PMID: 11782253 [PubMed - in process]